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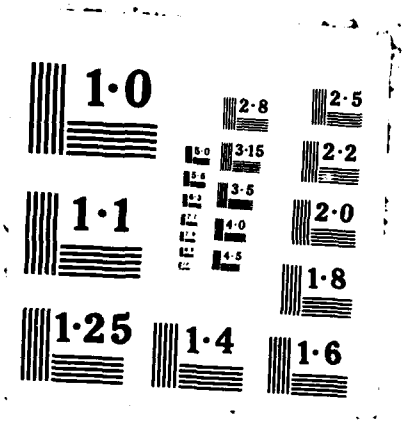
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ACTIVITY OF ORGANOPHOSPHATE ACID ANHYDRASE IN RANGIA CUNEATA

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RESEARCH DIRECTORATE

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Purified clam-digestive gland was used to individually test substrate solutions of DFP and Mipafox for organophosphate acid (opa) anhydrase activity. Results indicate three groups of molecular weight-estimates for substrate-specific enzymes within R. cuneata. When DFP was substrate, proteins in the 73,447 to 81,991 D and 20,157 to 43,547 D ranges were identified as opa anhydrases. With Mipafox as substrate, opa anhydrases ranging in weight from 105,026 to 138,286 D were discovered. This data suggests multiple enzymes within R. cuneata that are strictly characterized according to subtrate specificity and molecular weight.

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PREFACE

The work described in this report was authorized under Project No. 1C463721DE81, Chemical Decontamination Materiel. This work was started in August 1986 and completed in November 1986.

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ACTIVITY OF ORGANOPHOSPHATE ACID ANHYDRASE IN RANGIA CUNEATA

1. INTRODUCTION

Enzymes capable of hydrolyzing O,O-diisopropylfluorophosphate (DFP) and related acetylcholinesterase inhibitors, such as O-1,2,2-trimethylpropylmethylphosphonofluoridate (soman), 3,3-dimethylbutylmethylphosphonofluoridate (Dimebu), and N,N'-diisopropylphosphorodiamidofluoridate (Mipafox), have been reported in the tissues of many animals[1-5] and have recently been renamed as organophosphate acid (opa) anhydrases. Previously, two categories of DFP hydrolase enzymes, or DFPases, were partially characterized. "Squid-type" DFPase hydrolyzes DFP faster than soman,[2] is stable,[6] has a molecular weight of approximately 26,000 D,[7] is inhibited by manganous ion,[6] experiences no inhibition of DFP hydrolysis by Mipafox,[4] and is present in optic ganglia, giant nerve axon, hepatopancreas, and salivary gland of cephalopods.[8] "Mazur-type" DFPase is stimulated by manganous ion, hydrolyzes soman faster than DFP,[2] is dimeric with a molecular weight of approximately 62,000 D,[9] experiences inhibition of DFP hydrolysis by Mipafox,* and is unstable.[2] Activities resembling Mazur type are found in hog kidney, Escherichia coli, mammalian tissues, the protozoan Tetrahymena thermophila, and the clam Rangia cuneata.[1-3,9]

However, several DFPase sources have been shown to consist of more than one type of DFPase; hence the broader term, opa anhydrase, has been brought into use (DFPase Workshop, 1987). Five isoenzymes have been recognized in T. thermophila, ranging in molecular weight from 72,000 to 96,000 Daltons, that do not fit well into the classical squid- and Mazur-type definitions.[10] The use of Mipafox, which has been introduced to DFP hydrolysis studies as a tool for further enzyme identification and characterization, has provided evidence of mixtures of the DFPase types within a source. A previous study has found Mipafox to be a potent, reversible, competitive inhibitor of Mazur-type not squid-type DFPase, whether soman or DFP is used as substrate. Because of Mipafox's enzyme specificity, this study confirmed that various tissues, in particular, E. coli and squid, contain both DFPases.[4] One recent investigation[11] suggested the existence of both a DFPase- and Mipafox-hydrolyzing enzyme in clam digestive gland, when enzyme-mediated hydrolysis rates appeared to be additive of rates for DFP and Mipafox. The following study continues the investigation of opa anhydrase sources with a further look at Rangia cuneata.

*Landis, W.G., Chester, N.A., Haley, M.V., Johnson, D.W., and Tauber, R.M., "Mipafox as an Inhibitor of Tetrahymena thermophila - DFPase," unpublished paper.

2. METHOD AND MATERIALS

2.1 Tissue Preparation.

Rangia cuneata (clams) were collected in sediment samples from the Chesapeake Bay near Aberdeen Proving Ground and held in ambient water at 2 °C for several hours before processing. Homogenates, 33% clam tissue by weight in Hanks balanced salt solution (HBSS) (Difco), were prepared from digestive gland pooled from 30-50 individuals and stored at 4 °C. Before testing, the tissue was diluted with Hoskin's buffer (400 mM KCl, 50 mM NaCl, and 5 mM 1,3-bis [tris hydroxymethyl methylamino] propane [bis-tris-propane] in glass distilled water, pH 7.2), and centrifuged at 1500 rpm for 20 minutes, yielding a supernatant that was placed onto a column.

2.2 Column Preparation.

Column work was conducted at 4 °C using a 2.5- x 75-cm column. Hoskin's buffer as mobile phase was passed through a sephacryl S-300 superfine grade gel matrix (Pharmacia) at a flow rate of .483 mL/min. Collection time was set at 3 minutes per fraction or 1.45 ml per fraction. The following markers (Pharmacia Gel Filtration Calibration Kit, and Bovine Albumin, Sigma) were used for column calibration: blue dextran (2,000,000 D) ferritin (440,000 D), aldolase (158,000 D), BSA (67,000 D), and ovalbumin (43,000 D). Five milliliters of clam tissue extract was placed on the column, and fractions were collected and subsequently stored at -70 °C.

2.3 Enzyme Preparation.

Hoskin's buffer was used in the assays, and all chemicals were reagent grade. Two substrate solutions were individually tested for activity, 3.0×10^{-3} M of DFP and 3.0×10^{-3} of Mipafox, by adding them to a 30 °C water-jacketed glass beaker equipped with a magnetic stirrer. Substrate hydrolysis was quantified using a fluoride electrode attached to an Orion 901 microprocessor ion analyzer that recorded fluoride concentration at 1-minute intervals. After recording spontaneous hydrolysis, enzyme-mediated hydrolysis was measured following the addition of 300 µL of partially purified tissue extract taken from sets of 10 combined fractions. Later, those fractions in groups with the highest activities were individually tested with the same DFP and Mipafox concentrations. Determination of protein concentration of the fractions was measured using the Whitaker and Granum Ultraviolet Absorption Method.[5] Reaction rates were calculated using the Apple-computer program, DFPase2 (Appendix).

3. RESULTS

The figure presents the activities of the fractions found to have the highest rate of hydrolysis. When fractions were assayed with DFP, proteins at three distinct molecular weight ranges were found to have activity: 73,447 to 81,991 D, 20,157 to 43,547 D, and 198 to 299 D. As a result of using Mipafox as substrate, two entirely different molecular weight ranges of proteins were discovered: 105,026-138,286 D, and 2,112-3,331 D. Each fraction displayed elevated hydrolysis rates with both DFP and Mipafox as substrates. The denaturization of tissue at 70 °C for 20 minutes was found to destroy activity in the fractions whose molecular weight were below 20,000 D.

4. DISCUSSION

The digestive gland of Rangia cuneata contains substrate-specific enzymes which individually hydrolyze DFP and Mipafox. Three groups of molecular weight-estimates for heat labile enzymes allow the further characterization of clam's enzymatic activity. Previously, Rangia-DFPase was found to hydrolyze Soman greater than DFP and have a molecular weight of about 67,000-75,000 D with DFP as substrate. Exogenous Mn^{2+} stimulated soman but not DFP, hydrolysis by Clam DFPase.[3] Because of these characteristics, Rangia appeared to more closely resemble the Mazur-type enzyme. However, unlike other predominantly Mazur-type tissue extracts,[4,10] clam-digestive gland exhibits no inhibition of DFP hydrolysis by Mipafox.* Most of these extracts, taken from sources such as bacteria, protozoans, and mammals, have DFPases of molecular weight 62,000-75,000 Daltons. Our data presents a similar weight-range of 73,447-81,991 D, when DFP is substrate. This points to Mazur-type characterization; however, a second range of molecular weights, 20,157 to 43,547 D, was also determined with DFP as substrate. A weight of about 26,000 D is well within this range[8] and is associated with the squid-type enzyme, which suggests the existence of both enzyme types.

A third set of hydrolyzing enzymes was discovered to be Mipafox-specific. Proteins ranging in weight from 105,026 to 138,286 D were discovered when the assay used Mipafox as substrate. Although a DFP hydrolyzing-enzyme of 96,000 D has been reported in T. thermophila, it may be that these high molecular weight proteins require a classification of their own. The presence of a "Mipafoxase," helps to explain a trend of increasing hydrolysis rates when the unpurified digestive gland of clams was previously assayed using separate solutions of DFP and Mipafox and a third solution containing both.* Activity rates documented in the study were lowest with Mipafox as substrate, higher with DFP, and highest with both substrates present. This trend remains unique to R. cuneata.

It has become clear that a single tissue extract, such as digestive gland from R. cuneata, may contain multiple enzymes that can no longer be categorized according to their Mazur- or squid-type characteristics. The need for a wider classification of enzymes that hydrolyze DFP and related acetycholinesterase inhibitors has led to the renaming of these proteins as organophosphate acid anhydrases, or opa anhydrases (DFPase Workshop, 1987). In the case of the opa anhydrases of R. cuneata, each is now characterized according to substrate specificity and molecular weight.

*Chester, N.A., Anderson, P.S., and Landis, W.G., Mipafox As A Substrate for Rangia-DFPase, unpublished unclassified CRDEC report.

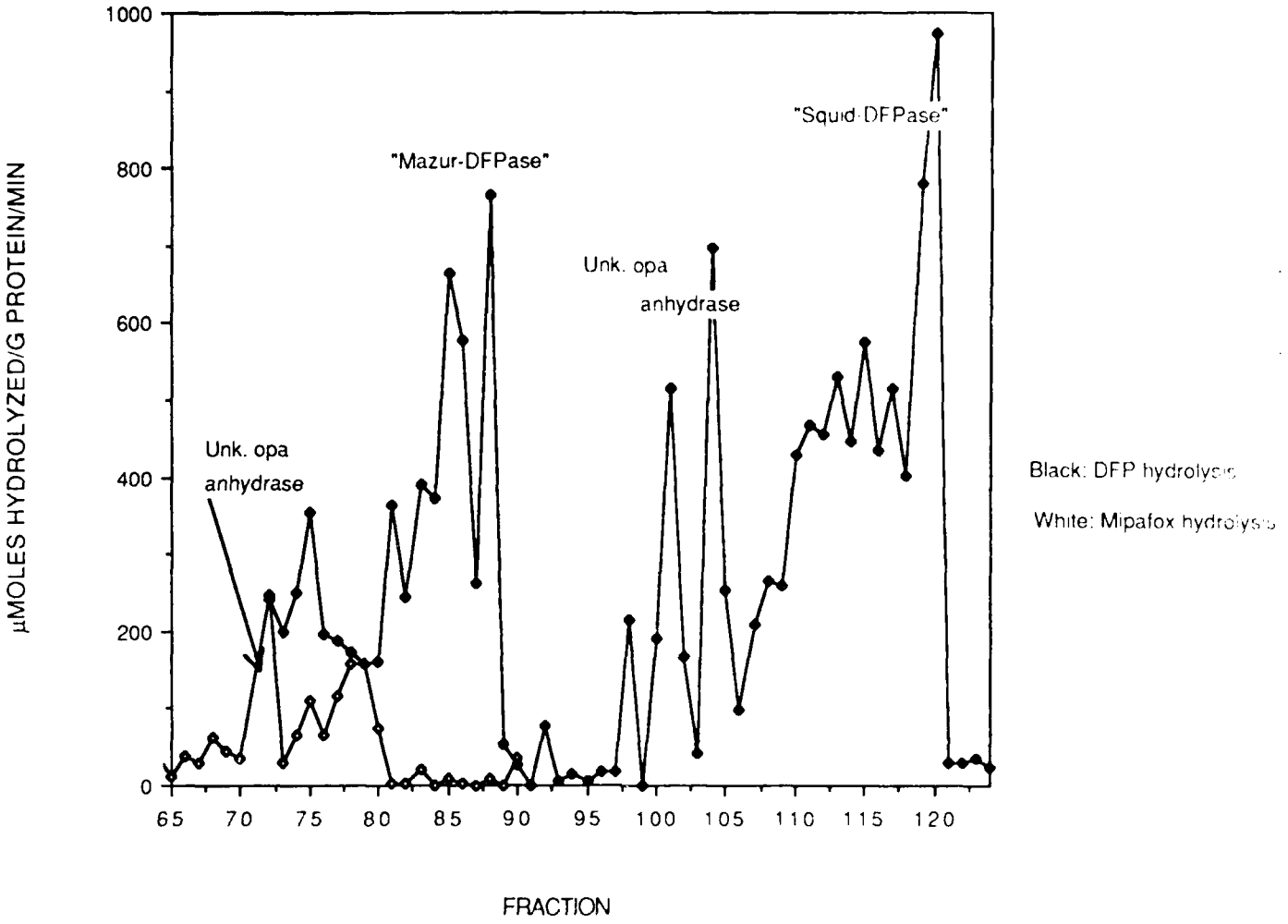

Figure 1. Hydrolysis of DFP and Mipafox by Clam

Opa anhydrases ranging from 73,447-81,991 D and 20,157-43,547 D were identified when DFP was used as a substrate, and 105,026 to 138,286 D when Mipafox was used as a substrate. These weights were grouped according to past molecular weights representative of Mazur-type DFPases, 62,000-75,000 D, and squid-type DFPases, about 26,000 D. Peaks at 138,286 and 43,547 D have not been previously characterized.

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APPENDIX

APPLE DEPASE2 PROGRAM

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```
1   PR# 3
2   INVERSE
5   PRINT  "**********  ***************DEPASE  PROGRAM  2  ****************
    ********************
10  PRINT "PROGRAM FOR CALCULATION OF ENZYMATIC HYDROLYSIS RATES OF "
20  PRINT "ORGANOFLUOROPHOSPHATES BY DEPASE AS MEASURED BY FLUORIDE
EVOLUTION"
30  PRINT "INPUTS REQUIRED ARE: TIME OF REACTION IN MINUTES, REACTION
VOL IN ML"
40  PRINT "VOLUME OF ENZYMATIC PREP IN ML, PROTEIN CONC. IN MG/ML, THE "
50  PRINT "STARTING AND FINISHING CONC. OF FLUORIDE IN uM, OR THE DELTA
IN  uM"
55  PRINT " AND THE SPONTANEOUS HYDROLYSIS RATE IN uM/MIN"
60  NORMAL
61  PRINT " INPUT SPONTANEOUS HYDROLYSIS"
62  INPUT S
70  PRINT "INPUT TIME, REACTION VOLUME"
80  INPUT T,V
90  PRINT "INPUT VOL. ENZYMATIC PREP, PROTEIN CONC."
100     INPUT E,P
105     S1 = S * T
110     T1 = 60 / T
120     V1 = 1000 / V
130     E1 = 1 / E
131     PRINT "IF INPUT IS START AND FINISH CONC. KEY 0, IF DIFFERENCE KEY
1"
132     INPUT W
133     IF W = 0 THEN  GOTO 140
134     IF W = 1 THEN  GOTO 135
135     PRINT "INPUT DELTA uM"
136     INPUT CT
137     GOTO 161
140     PRINT "INPUT START FLUORIDE, FINISH FLUORIDE"
150     INPUT C1, C2
160     CT = C2 - C1
161     CC = CT - S1
170     R1 = CC  * (E1 * T1) / V1)
171     PRINT "RUN NUMBER  ?"
172     INPUT W
174     PR#  1
175     PRINT    "**********************************
**********************************
178     PRINT "RUN NUMBER  ";W;"SPONTANEOUS HYDROLYSIS RATE=";S
179     PRINT "TIME=";T;"VOL=";V;"ENZ PREP=";E;"PROTEIN  ";P;"uM  ";CC
180     PRINT R1;"uMOLES HYDROLYZED /MI/HR"
190     R2 = R1 / 60
200     PRINT R2;"uMOLES HYDROLYZED /MI/MIN"
210     IF P = 0 THEN  GOTO 265
220     P1 = 1000 / P
230     R3 = R1 * P1
240     PRINT R3;"uMOLES HYDROLYZED / PROTEIN/HR
```

```
250    R4 = R3 / 60
260    PRINT R4;"uMOLES HYDROLYZED /G PROTEIN/MIN"
265    PRINT    "**********************************************
**************************"
266    PR# 0
267    PR# 3
270    PRINT "KEY 1 IF ONLY THE START AND FINISH CONC. CHANGE'
280    PRINT "KEY 2 IF TIME, VOL, PROTEIN CONC., OR VOL. ENZYME PREP
CHANGES"
285    PRINT "KEY 3 IF THE RATE OF SPONTANEOUS HYDROLYSIS ALSO CHANGES"
290    PRINT "KEY 4 IF YOU ARE FINISHED"
300    INPUT Z
310    IF Z = 1 THEN GOTO 131
320    IF Z = 2 THEN GOTO 70
330    IF Z = 3 THEN GOTO 61
335    IF Z = 4 THEN GOTO 390
390    END
```

END

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MARCH, 1988

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